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Investigation Into Roseburia Intesinalis For Optimal Growth And Survivability Conditions For Potential Probiotic Use

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INVESTIGATION INTO *ROSEBURIA INTESINALIS* FOR OPTIMAL GROWTH AND SURVIVABILITY CONDITIONS FOR POTENTIAL PROBIOTIC USE

by

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_____

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DEDICATION

I dedicate this thesis to my grandmother, Madeline Jamroz, whom encouraged and supported the continuation of my education and who is no longer here to watch me finish. Also, to my parents, Timothy and Cathy Felczak, for always giving me their advice and support.

ACKNOWLEDGMENTS

I would like to acknowledge and graciously thank Dr. Kevin Zhou for his continued support and advice during my thesis research. I also would like to thank Wenjun Zhu for his help throughout my project; I couldn't have done this without him. Also, to all the members in Dr. Zhou's lab for their continued help and advice. Finally, to Dr. Ahmad Heydari and Dr. Cathy Jen for serving on my masters committee.

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LIST OF ABBREVIATIONS AND ACRONYMS

BMI: Body Mass Index
DALYs: Disability Adjusted Life Years
GI: Gastrointestinal
GF: Germ Free
CONV-R: Conventionally Raised
GSIS – Glucose Stimulated Insulin Resistance
R. Intestinalis: Roseburia Intestinalis
Spp: Species
IBS: Irritable Bowel Syndrome
UC: Ulcerative Colitis
SCFAs: Short Chain Fatty Acids
HFD: High Fat Diet
IMO: Isomaltooligosaccharide
BF: Benefiber
GG: Guar Gum
CMC: Carboxy Methyl Cellulose
Oligo: Oligo-Chitosan 95%
XOS: Xylooligosaccharide
FOS: Fructooligosaccharide

INTRODUCTION

Obesity and Diabetes

Since 1980 the prevalence of obesity (Body Mass Index (BMI) ≥30 kg/m^2) has nearly doubled from 13% of adults aged 20+ to todays average of over 24% percent of adults aged 20+, worldwide (1). Adverse metabolic effects such as increased blood pressure, triglycerides, cholesterol, and insulin resistance are often associated with obesity. Risk of certain cancer incidences such as breast, prostate, endometrium, kidney, colon, and gall bladder have shown to increase as BMI increases as well. An approximate 35.8 million (2.3%) of global disability adjusted life years (DALYs) are caused as a result of being overweight/obese and more than 2.8 million people die each year as a direct result (1) .

The onset of type 2 diabetes begins when the body develops sensitivity to insulin. Insulin is an important hormone secreted by the pancreas' beta cells to facilitate the transfer of postprandial blood glucose from the blood stream into cells. If more sugar is consumed than the body needs, insulin aids in storing the excess glucose in the liver. Overtime, continual exposure to an abundance of glucose can cause the beta cells to become desensitized causing a drastic decrease or potentially stopping the production of insulin all together. Without insulin, cells cannot uptake glucose from the blood stream to be used as fuel and the body will need to enter ketosis to maintain an energy supply. Prolonged exposure to excess sugar in the blood stream and ketosis will eventually lead to multiple organ failure and, if untreated, death (2).

Research has found that genes play a larger role in the connection between the immune system, metabolism, and environment than previously thought. This complex

connection has shown to alter the outcome of body weight, influencing energy expenditure, appetite, and many finite metabolic functions (3-5). Despite these metabolic predispositions that may help to facilitate obesity, genetics alone cannot explain the increased incidence of obesity associated with the consumption of a Western diet (e.g. high in glucose, fructose, sucrose, saturated fats, and empty carbohydrates).

Acetate Consumption and Increased Insulin Secretion

Short chain fatty acids (SCFAs) are the main products of fermentation. Acetate, propionate, and butyrate are produced when dietary fibers are fermented by anaerobic colonic microbiota (6). Recently research has looked at the role these SCFAs play in the health of our gut and has found a link between increased acetate production and obesity. A study conducted by Perry *et al.* examined glucose-stimulated insulin secretion (GSIS) during a hyperglycemic clamp. Perry and her team wanted to examine whether increases in acetate turnover drove an increase in GSIS. To do this they performed hyperglycemic clamps in chow fed rats given intra-arterial infusions of acetate matching that of the whole body turn over of acetate measured in high fat diet (HFD) fed rats. The rats fed the acetate infusion showed similar increases in GSIS to that of the rats fed the HFD. Going further, when replacing the butyrate turnover to levels compared to that in the HFD rats they found there was no effect on GSIS. This research suggests increased acetate turnover has the ability to drive insulin secretion (7).

The Gut Microbiota

The human gastrointestinal (GI) tract's ecosystem is primarily composed of bacteria. The colon has the highest density of cells that has ever been demonstrated in any

ecosystem (1011-1012/mL contents). Bacteroidetes (48%) and Firmicutes (51%) are two phylums of bacteria that make up the majority of the inhabitants (8). The human gut microbiota consists of 10^{13} to 10^{14} microorganisms. These bacteria are mutually beneficial for the host organism, utilizing their ability to break down ingested polysaccharides into monosaccharide form and further fermenting them into short-chain fatty acids. Since humans evolved without the enzymes to carry out this function on our own, this mutualistic relationship allows the host organism to acquire carbon and energy while the microbes are protected in an anoxic environment and are provided with glycans as a fuel source (9).

In 2004, Backhed *et al.* were among the first to investigate the potential of the gut microbiota as an environmental factor that could be regulating fat storage through an integrated host pathway. Backhed and his team experimented using male B6 mice 8- to 10- weeks of age. They compared B6 mice raised in the absence of microorganisms (GF) to that of mice containing a microbiota since birth (CONV-R). Their findings showed that although CONV-R mice consumed 29% less standard rodent chow than the GF mice, CONV-R mice were observed to have 42% more total body fat, as well as 47% greater weight in epididymal fat pads. In addition, Backhed and his collogues found that within 14 days of conventionalization of GF B6 mice (using normal microbiota obtained from the cecum of CONV-R mice) there was a 60% increase in body fat content and an increased insulin resistance regardless of having reduced food intake. Backhed and his team discovered the mechanism causing the additional body fat was an increase in the absorption of monosaccharides promoted by the gut microbiota, suggesting that the gut

microbiota is a crucial environmental factor affecting energy accumulation and storage in the host (10).

When comparing the mouse gut microbiota to that of a human it has been found that the majority of species are unique to the mouse, but they are comparable at the phylum classification with Firmicutes and Bacteroidetes dominating (11). Since the composition of the mice microbiome is inherited from the mother, Ley *et al*. wanted to see how obesity could affect the diversity occurring in the gut. C57BL/6J *ob/+* mothers and their offspring (*ob/ob, ob/+*, and +/+) were all fed the same polysaccharide rich diet. Ley and his team discovered that when comparing the obese mice to the lean, the *ob/ob* animals showed a 50% reduction in the quantity of Bacteroidetes and an equivalent increase in Firmicutes (11). This finding demonstrates how obesity seems to be affected by the shifting of the dominating phylum in the gut, but was unsuccessful at showing further insight into the composition and distribution of the species of each phylum of these bacteria.

Probiotics are live yeast and bacteria that have been thought to be advantageous for health, especially for the health of the gut, and can be found in foods, as dietary supplements and in skin care creams. The World Health Organization defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (12). In recent years prebiotics have become an emerging area of interest for their potential effect on the gut microbes. Prebiotics are dietary fibers that have been shown to be beneficial fuel source for the growth and reproduction of the bacterial microflora. The Food and Agriculture Organization of the United States (FAO)

defined a prebiotic as “a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota” (13).

Phylum Fimicutes

The phylum Firmicutes is the largest group of bacteria because it is dominated by very successful and diverse generas. Firmicutes mainly stain gram-positive due to their thick peptidoglycan cell made of protein and carbohydrates that give structure and protection from osmotic stress. There is 2,850 species to date within the phylum Firmicutes separated into seven classes: the Erysipelotrichia, the Negativicutes, the Limnochordia, the Tissierellia, the Thermolithobacteria, the Clostridia and the Bacilli. Firmicutes are further divided and categorized by their G+C nucleotide ratio. The percentage of G+C determines if a group is considered a “low G+C” or a “high G+C” Firmicutes. Some Firmicutes have the ability to form an endospore. An endospore is a resistant cell usually produced under stressful conditions. Unlike the oxygen loving Bacilli, The Clostridium class contains many anaerobic species. These species have different methods for obtaining energy without oxygen, mainly through fermentation. Recently, research has been focused on the multiple species within the Clostridium class that is anaerobic and has been found to be butyrate-producing, an important and preferred energy source for the colonic epithelial cells and have shown to help maintain colon health (14). The phylum Firmicutes is a used in many different industries including ecology, medicine, agriculture and food production to name a few, making them a major area for investigation (15).

Roseburia intestinalis

Species *Roseburia intestinalis* (*R. Intestinalis)* are members of the phylum Firmicutes, class Clostridia, order Clostridiales, Family Lachnospriaceae, and genus *Roseburia (16).* The genus *Roseburia* was named in honor of Theodor Rosebury, a bacteriologist who has been called the "Grandfather of Modern Oral Microbiology" (17). Along with RI, there have been five species discovered in this genus. RI and the other species in the *Roseburia* genus are characterized by being Gram-positive and anaerobic, having a curved rod shape, low G+C content, have the ability to convert SCFAs to produce butyrate and exhibit motility by means of subterminal flagella (18).

Roseburia clusters have been predicted to be around 3 to 15% of the total bacterial count found in a healthy gut (19). Recent research has found a link between *Roseburia* spp. and overall gut health, including irritable bowel syndrome (IBS), inflammatory bowel disease, and colon cancer (20). Van den Abbeele *et al.* developed a dynamic in vitro gut model that has the ability to stimulate luminal and mucosal gut microbes. These researchers found that RI specifically colonized mucins and the butyryl-CoA:acetate-CoA transferase gene sequences belonged to different species in the luminal than those colonizing the mucins. These findings suggest that mucosal butyrate producers may be helpful in treating GI diseases by increasing the butyrate bioavailability because the butyrate is produced close to the epithelium (21).

Specifically in ulcerative colitis (UC), studies have found a decrease in the prominent butyrate producers, such as RI, has been correlated to a reduction in short chain fatty acid concentrations. Kumari *et al.* wanted to look at the interaction between butyrate producing bacteria and butyrate concentrations in UC patients when compared to that of control individuals. Kumari and colleagues discovered that, when compared to the

control, the nonhealthy patients exhibited decreased butyrate concentrations and well as decreased butyrate producers. They suggest the reduction of butyrate producers and in turn reduction in butyrate can lead to a decreased supply of energy to the colon and can eventually lead to GI problems (22).

OBJECTIVE

The objective of the current study was to determine the optimal growth conditions for *R. Intestinalis* by adding different concentrations of SCFAs and prebiotics, and to determine the effects of environmental factors including pH, bile salts, and antibiotics, as well as effects storage conditions on the survivability of *R. Intestinalis*.

MATERIALS AND METHODS

General Culturing

Roseburia intestinalis was purchased from DSMZ (Leibniz Institute DSMZ, Germany). General culture was done in PYG media with the addition of SCFAs according to DSMZ instructions (23) under anaerobic conditions using Hungate roll tube method, N_2 gas, and anaerobic indicator Resazurin (ACROS, Fair Lawn, NJ) at 0.5mg/L. *R. Intestinalis* was transferred into PYG Hungate tubes using 1 mL syringe with 26G needle (BD, Franklin Lakes, NJ) and incubated at 37°C.

PCR Amplification and Gel Electrophoresis

DNA extraction was done using QIAmp DNA Mini Kit (Qiagen, CA) according to the manufacture's instructions. Primers were designed using the NCBI database from *R. Intestinalis's* 16s rDNA (Table 1). Eppendorf© thermocycler (Hauppauge, NY) was used to run the PCR. 12.5 µl Sigma ReadyMix™ RedTaq© PCR Reaction Mix (Foster,

CA) 0.1µM of each primer, 2 µl DNA template, and PCR water was added to a total volume of 25 µL. Denaturation was carried out at 94°C for five minutes, 60°C for 2 minutes and 72°C for 2 minutes followed by 29°C cycles of 94°C for 2 minutes 60°C for 30 seconds and 72°C for 2 minutes for amplification, and a final cycle step of 72°C for 10 minutes (24). A 1.5% agarose gel was prepared in 1x Tris-Borate-EDTA buffer and was used for gel electrophoresis. Thermo Scientific© GeneRuler Ultra Low Range DNA Ladder (Carlsbad, CA) was used as a standard. SYBR© Green stain (Carlsbad, US) under Bio-rad© Imager (Hercules, CA) was to examine final results.

Growth Conditions

Short Chain Fatty Acids

R. Intestinalis was inoculated into Hungate tubes of PYG with varying SCFAs. A SCFA mixture of 33 mM acetate (Sigma Aldrich, St. Louis, MO) with 9 mM propionate (Sigma Aldrich, St. Louis, MO) suggested by the manufacturer (Leibniz Institute DSMZ, Germany) was used for routine culturing. Additionally, PYG tubes containing 33 mM, 66mM, and 132 mM acetate, alone, were tested. Tubes were incubated at 37 °C for six hours with turbidity measurement taken every half hour. Turbidity value was determined using a SIEMENS© turbidity meter (West Sacramento, CA) at 595 nm.

Prebiotics

R. Intestinalis was inoculated into Hungate tubes of PYG with various prebiotics. 0.5% each of isomaltooligosaccharide (Raw Indulgence LTD, NY), Benefiber (Parsippany, NJ) guar gum (Good Gut Solution Company, San Diego, CA), carboxy methylcellulose GlaxoSmithKline, Moon Township, PA) oligo-chitosan 95% Xian Lukee

Bio-Tech Co., China), galactooligosaccharide (Quantum HI-tech Biological Co. Ltd, China), xylooligosaccahride (Quantum HI-tech Biological Co. Ltd, China), fructooligosaccharide (Quantum HI-tech Biological Co. Ltd, China), and the positive control of glucose (Acros, NJ)were used. Tubes were incubated at 37 °C for six hours with turbidity measurement taken every half hour. Turbidity value was determined using SIEMENS© turbidity meter at 595 nm.

Survivability Conditions

pH

R. Intestinalis was inoculated into Hungate tubes of PYG with varying pH. pH 2, 3, 4, 5, 6, 7, 8, 9, and 10 were tested. PYG's standard pH is 7.04. Cysteine (Fischer Scientific, Fair Lawn, NJ) was added to lower pH while Sodium Bicarbonate (Mallinckrodt Chemicals, Phillipsburg, NJ) was added to increase the pH. pH was found using Oakton© pH 700 Bench Meter (Vernon Hills, IL). Tubes were incubated at 37 °C for 24 hours with turbidity measurement taken at half hour, one hour, four hours and 24 hours to measure survivability as well as find the optimal pH for growth. Turbidity value was determined using SIEMENS© turbidity meter at 595 nm.

Bile Salts

R. Intestinalis was inoculated into Hungate tubes of PYG with varying concentrations of bile salts (Fisher Science Education bile salts, Nazareth, PA). 0.1 g/L, 0.5 g/L, 1.0 g/L, and 5.0 g/L were used. A negative control containing no bile salt was used for comparison. Tubes were incubated at 37 °C for four hours with turbidity measurement taken at time zero, two hours and four hours to mimic the amount of

exposure in vivo. Turbidity value was determined using SIEMENS© turbidity meter at 595 nm. At 4 hours, samples were taken from each tube and serial diluted using phosphate buffer for further plating using PYG agar (Sigma Aldrich, St. Louis, MO) 20 µL of each bile salt concentration and negative control were plated onto Fischer Scientific 100 mm x 15 mm plates and put into anaerobic chamber BBL Gas Pak before being incubated at 37 °C for 24 hours. After growth CFU was counted to check viability.

Antibiotics

R. Intestinalis was inoculated onto a 48-well plate (Costar, Corning, NY) containing PYG with various antibiotics. 10 µg/mL and 20 µg/mL of each Azithromycin (Sigma Aldrich, St. Louis, MO), Sulfamethoxzaole (Sigma Aldrich, St. Louis, MO), Oxytetracyclin (Sigma Aldrich, St. Louis, MO), Trimethoprim (Sigma Aldrich, St. Louis, MO), Erythromycin (Sigma Aldrich, St. Louis, MO), and Ciprofloacin (Sigma Aldrich, St. Louis, MO) were used. A blank of just PYG without *R. Intestinalis* or antibiotics, and a negative control containing PYG and *R. Intestinalis* without antibiotics was used for comparison. The plate was incubated at 37 °C for six hours in Perkin Elmer HTS 800 Bioassay Reader, and absorbance measurement was taken every hour at 595 nm.

Characterization Methods

Generation Time

R. Intestinalis was inoculated into Hungate tubes of PYG and incubated at 37 °C. 20 µL sample was taken at time zero and four hours and plated onto agar plates and put into anaerobic chamber before being incubated at 37 °C for 24 hours. After growth CFU

was counted and used in the generation time equation to establish *R. Intestinalis's* generation time (below) where log is the logarithm of the base (25).

$$\text{Generation Time} = \frac{\text{Duration (minutes or hours)}}{(3.3)(\log(\text{Final Concentration}) - \log(\text{Initial Concentration}))}$$

Growth Curve

R. Intestinalis was serially diluted and inoculated onto a 48-well plate (Costar, Corning, NY) containing PYG. The plate was incubated at 37 °C for 12 hours in microplate reader and absorbance measurement was taken every hour at 595 nm. 20 µL sample was taken after lag phase during the exponential growth phase determined by increasing absorbance values. CFU were counted and plotted against absorbance using Microsoft Excel (Microsoft Corporation, Redmond, WA) to generate an absorbance curve.

Processing Methods

Freeze Drying

R. Intestinalis was inoculated into 2.5 L of PYG and incubated at 37 °C for 12 hours. Media was centrifuged and excess media was disposed of. A 10% sucrose media solution was added for protection. Liquid nitrogen was used to flash freeze the RI and it was stored in -80° C freezer overnight. Labconco Triad freeze dryer was used. The sample was kept in the freezer dryer for 3 days until sample was completely dried. Freeze-dried samples are stored in -20° C for future use. Freeze-dried samples were

reconstituted into PYG media and incubated at 37° C for one hour before being plated using PYG agar to ensure the bacteria is still viable after freeze drying method.

Encapsulation

Freeze-dried *R. Intestinalis* was encapsulated according to method from Sohail *et al. (26)* (adapted for use by colleague Jiarun Cui). *R. Intestinalis* was encapsulated using 1% Sodium Alginate (Acros NJ), 1% Casein (Hard Eight Nutrition, Henderson, NV) , 0.5% prebiotic (guar gum) to 3% bacteria. Solution is dropped into Calcium Chloride (Sigma Aldrich, St. Louis, MO) to solidify the bacterial solution. Beads were put under hood and allowed to dry overnight. To check viability, encapsulated beads and a freeze-dried sample (no encapsulation) were exposed to physiological digestive conditions. Beads and freeze-dried samples were added to a 20 mg/mL Pepsin (Sigma Aldrich, St. Louis, MO) HCL (Sigma Aldrich, St. Louis, MO) solution and incubated at 37° C for one hour to mimic gastric digestion. After one hour, the mixture is centrifuged and the Pepsin HCL is disposed of. A 33mg/mL solution of Enteric Digestive Fluid was immediately added incubated at 37° C for two hours to mimic enteric digestion. Beads are homogenized using to release the bacteria and plated using PYG agar. Plates were put into anaerobic chamber before being incubated at 37 °C for 24 hours. After growth CFU was counted to check viability and to compare survivability in each storage condition.

RESULTS AND DISCUSSIONS

Gel Electrophoresis

Figure 1 shows the gel electrophoresis ran with the *R.intestinalis* DNA primers against the low range DNA ladder. Only primer one showed a visible band between 150 bp and 200 bp, whereas the band is slightly closer to the 150 bp band in confirmation with the 159 bp primer one has. This gel electrophoresis is able to show the DNA that was extracted and amplified does belong to *R. intestinalis.*

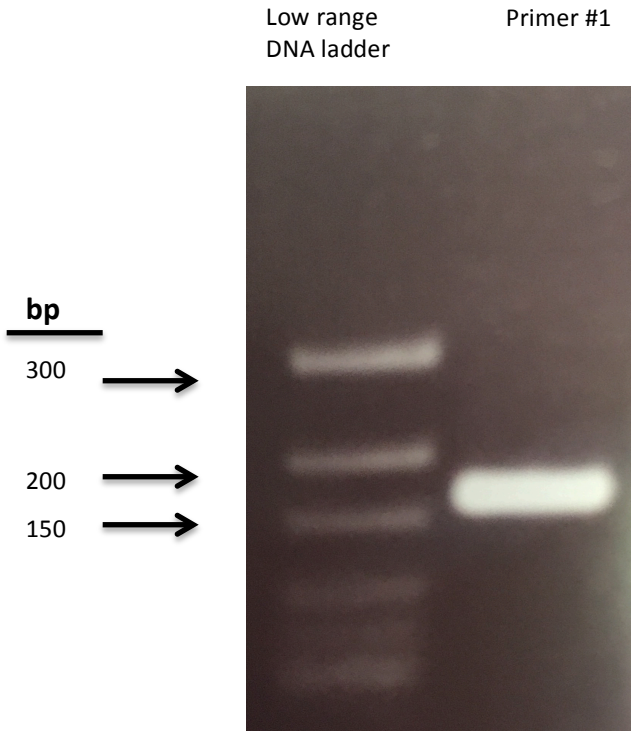

Figure 1- Agarose gel showing the amplification and identification of *R.intestinalis* 16S rDNA when compared to a low range DNA ladder

Short Chain Fatty Acids

From literature research *R.intestinalis* has been shown to convert acetate to butyrate, a desired end product. Figure 2 is examining the growth of *R.intestinalis* at different concentrations of acetate when compared to the literature SCFA recommendation for growth, an acetate (33 mM) and propionate (9 mM) mixture, to see if *R.intestinalis* had the ability to grow in acetate alone and at what concentration yielded the most growth. From time 30 minutes to 150 minutes all SCFA conditions showed similar growth as a result of the lag phase. At 180 minutes the SCFA mixture and 66 mM acetate begin to increase growth while 33 mM acetate and 132 mM acetate do not show increased growth at the same rate. 132 mM acetate seemed to cause a longer lag time before growth was seen at 270 minutes. Too much acetate in the media may have made it harder for the *R.intestinalis* to utilize the other crucial nutrients in the media increasing the time it took to reach their exponential growth phase. SCFA mixture and 66 mM acetate were found to be very comparable for the optimal growth condition, with SCFA showing slightly increased growth near the end. Since *R.intestinalis* converts acetate through the butyryl-CoA:acetate-CoA transferase gene, it was important to see how different concentrations of acetate alone could affect growth when compared with the literature recommendation for future studies trying to optimize the butyrate conversion potential.

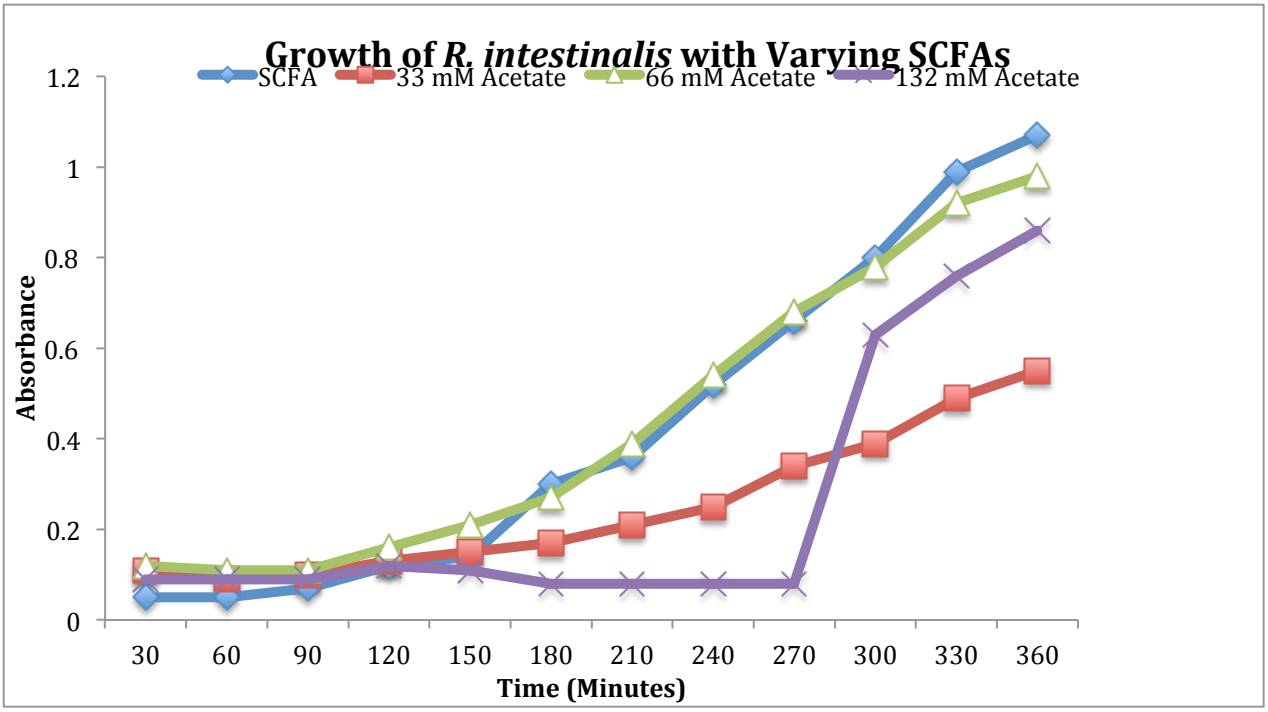

Figure 2- Growth of *R.intestinalis* at varying concentrations of acetate and compared to a SCFA mixture of acetate and propionate to identify the optimal condition.

Prebiotics

Figure 3 examines the effect of prebiotics on the growth of *R.intestinalis* as compared to glucose alone. Table 1 gives the full names of the prebiotics used and a short description of what they are. As shown in Figure 3, the lag phase lasted from 30 minutes to around 150 minutes in accordance with previous results. At 180 minutes three prebiotics begin to show growth similar to glucose, guar gum, isomaltooligosaccharide, and fructooligosaccharide, respectively.

Upon further research into these prebiotics it was discovered that guar gum is composed of galactose and mannose, which are both epimers of glucose. Galactose and mannose have the same chemical formula as glucose but are stereochemically different at carbon-4 and carbon-2 configuration, respectively. Because of this, *R.intestinalis* most

likely is able to utilize the guar gum in the same manner at which it utilizes glucose. Isomaltooligosaccharide is an oligomer of glucose. *R.intestinalis* would be able to utilize the IMO exactly the same as glucose but it may take more energy to break the bonds to use the glucose and in turn cause slightly less growth than glucose alone.

Table 1 – Full names and Descriptions of prebiotics used to see which provided optimal growth to *R.intestinalis*

Prebiotic	Description
IMO = Isomaltooligosaccharide	• Short chain carbohydrates • Digestion resistant • Made from starch
BF = Benefiber	• By product of wheat dextrin and inulin
GG = Guar Gum	• Made from guar beans • Galactomannan • Polysaccharide composed of galactose and mannose
CMC = Carboxy Methyl Cellulose	• Derivative of cellulose • Often used as sodium salt
Oligo = Oligo-Chitosan 95%	• Made from shells of crustaceans • β- (1-4) D-glucosamine links
GOS = Galactooligosaccharide	• Produced from enzymatic conversion of lactose • Comprised of galactose units
XOS = Xylooligosaccharides	• Polymers of xylose sugars
FOS = Fruitooligosaccharides	• Produced from inulin degradation • Resistant to hydrolysis by amylase & digestive enzymes
Glucose	• Simple sugar, circulates as blood sugar in mammals

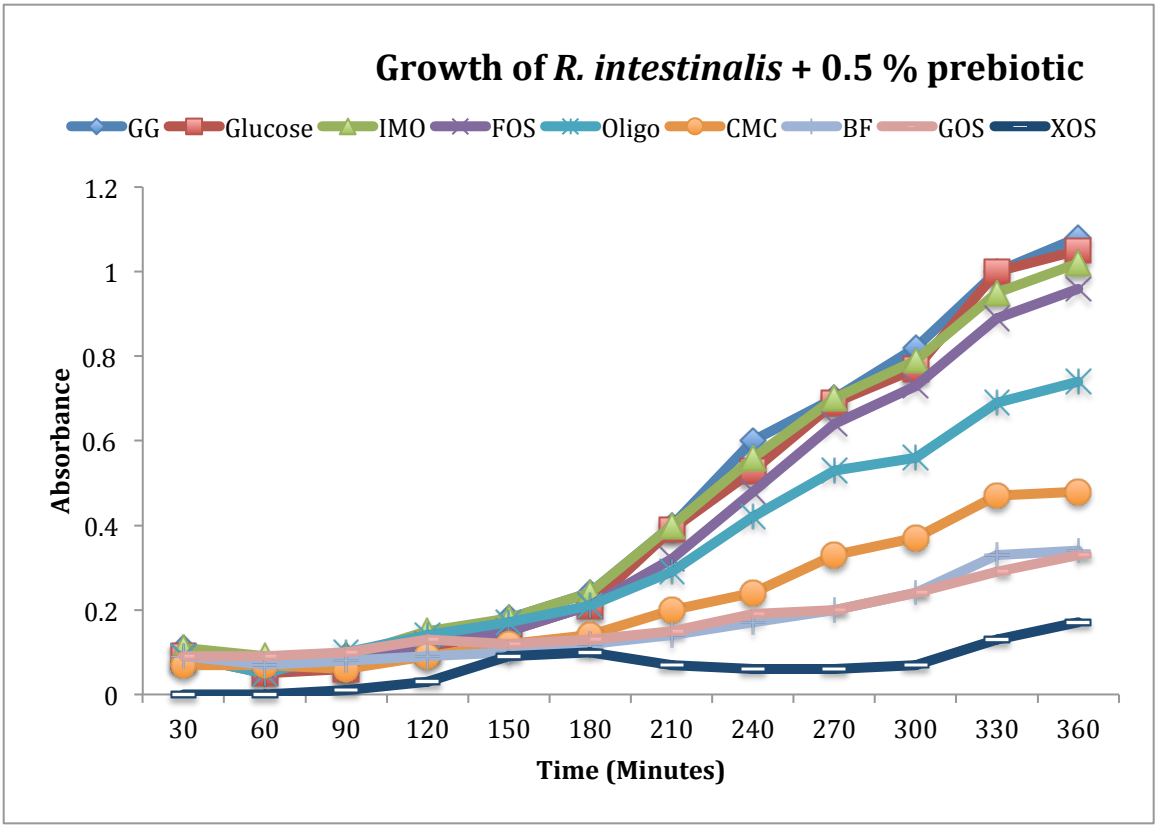

Figure 3 – Effects of prebiotics on the growth of *R.intestinalis* as compared to glucose alone.

pH

Error! Reference source not found. shows the optimal growth of *R.intestinalis* at varying pH. Initial absorbance taken at time zero shows a higher absorbance as the pH increases due to the addition of sodium bicarbonate. pH 2, 3, 4, and 5 all had very similar growth making the plot line overlap for each. When tested these pH plot line stayed constant and were not found to be the optimal growth condition. pH 10 had the highest absorbance initially due to the addition of the most sodium bicarbonate. pH 10 was also found to inhibit the growth of *R.intestinalis* and the plot line stayed constant. At pH 6 growth begins but was not found to be the optimal condition. At pH 7, 8, and 9 growth of

R.intestinalis was found to be optimal with pH 8 showing the highest absorbance during the exponential phase. Freeze dried bacteria was used and most likely caused an increase in lag time. At four hours there was not a significant difference between the varying pH so additional time was given to find the optimal pH for growth.

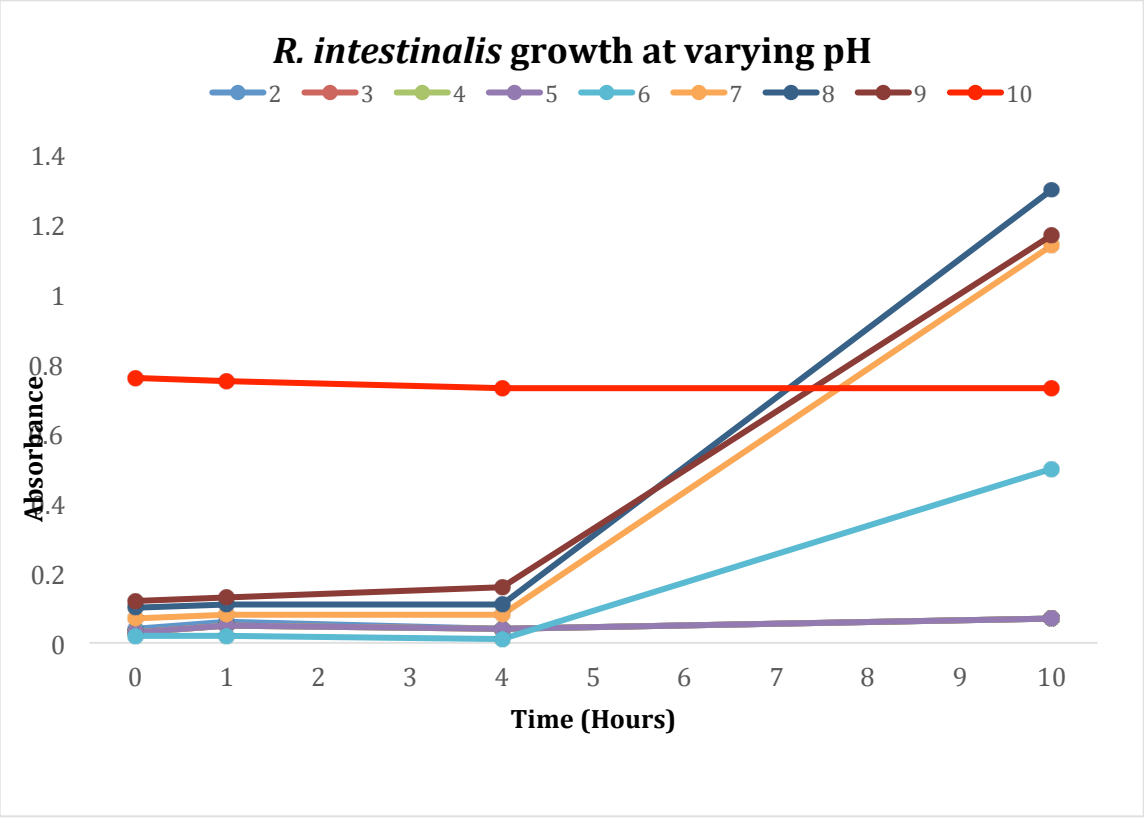

Figure 4 – Growth of R.intestinalis at varying pH 2, 3, 4, 5, 6, 7, 8, 9 and 10

Bile Salts

Figure 5 is examining the effect of various concentrations of bile salts on the survivability of *R.intestinalis.* As a result of the digestion process, these bacteria will be vulnerable to exposure of bile salts and to be a successful probiotic they will have to withstand the 400 mL to 800 mL that is produced daily (27). From this figure it can be examined that, when compared with the negative control, *R.intestinalis* shows the best survivability at the lowest concentration, 0.1 g/L. Bacteria was only plated for 240

minutes to mimic the exposure time that would occur in vivo. As the concentration of bile salts increased the survivability of *R.intestinalis* decreased. Although there is not a significant amount of growth occurring at these concentrations of bile salts, it cannot be assumed the *R.intestinalis* are no longer viable. To check the viability of the *R.intestinalis* at the different concentrations of bile salts a sample of each concentration was plated with PYG agar at time zero and 240 minutes (Figure 6). CFU counts showed that for each 0.1 g/L, 0.5 g/L, and 1.0 g/L the *R.intestinalis* were still viable and showed growth when compared to the CFU count at time zero. Concentration 5g/L showed no growth when compared with time zero. These results showed that although increasing the concentration of bile salts did not increase the growth of *R.intestinalis,* plating the bacteria to further check their viability showed they could survive in concentrations up to 1.0 g/L

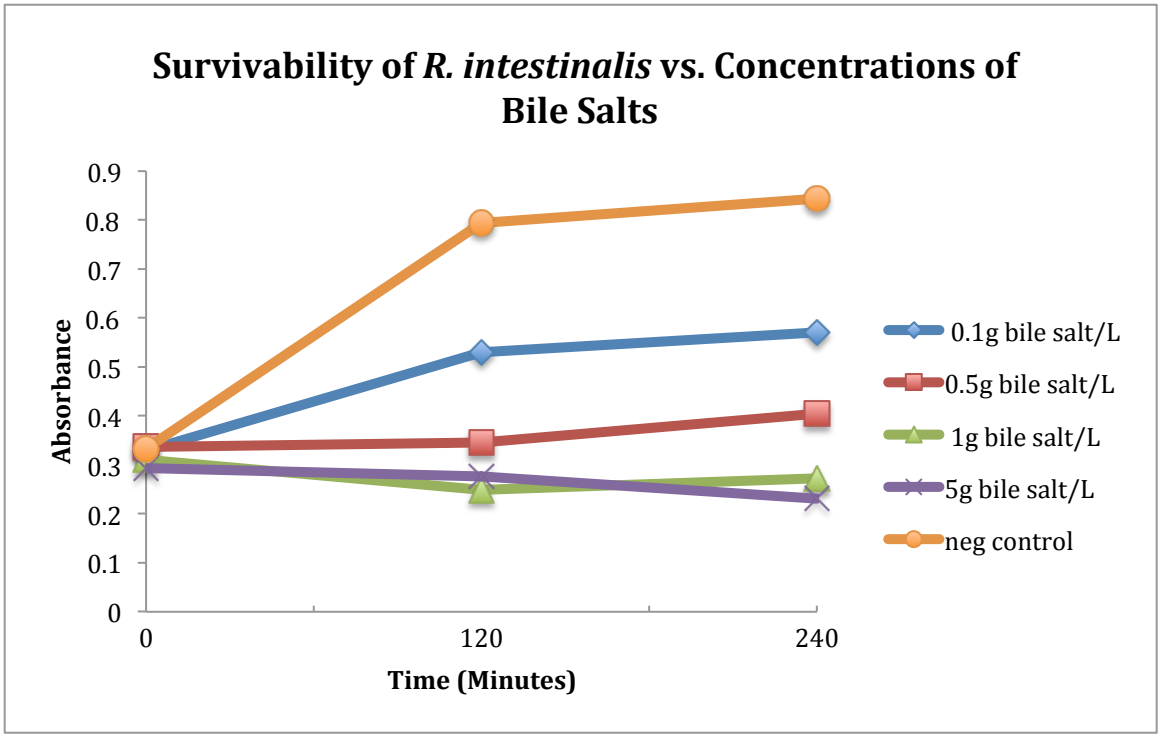

Figure 5 –Survivability of *R.intestinalis* at various concentrations of bile salts.

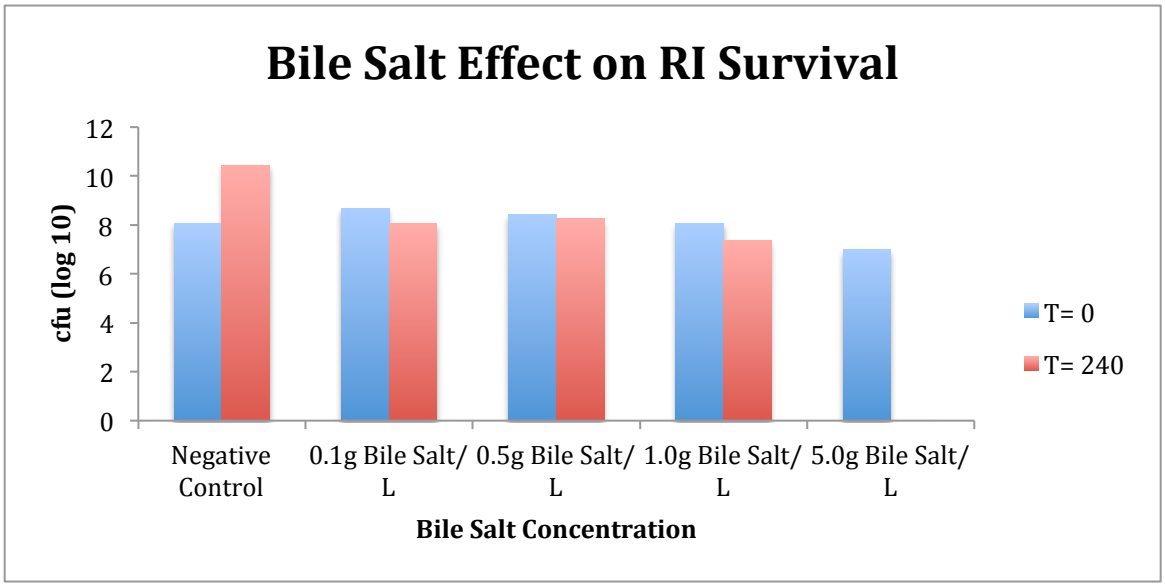

Figure 6 – Viability CFU check of *R.intestinalis* after incubated with various concentrations of bile salts (0-5.0 g/L).

Antibiotics

Various antibiotics at 10 µg/mL and 20 µg/mL were tested for their effect on the survivability of *R.intestinalis* for the potential use as a probiotic to repopulate the gut while on antibiotics. Azithromycin, Sulfamethoxzaole, Oxytetracycline, Trimethoprim, Erythromycin, and Ciprofloacin were used. Table 3 gives the mode of action of each antibiotic used.

Table 2 - Descriptions of antibiotics modes of action tested on *R.intestinalis*

Antibiotic	Description
Azithromycin	• Broad but shallow • Treats **some** gram + / -
Ciprofloxacin	• In the class of fluroquinolones • Many bacteria have developed resistance due to widespread use to treat minor infections
Erythromycin	• Treats bacteria responsible for causing infections of the skin • Displays bacteriostatic activity but mechanism is not yet understood
Trimethoprim	• Mainly used for treatment of bladder infections • Not recommended for treatment of anaerobic infections
Oxytetracycline	• Broad spectrum antibiotic that effects bacteria's ability to produce essential proteins • Resistance is on the rise
Sulfamethoazole	• Mechanism of action – prevents folic acid synthesis • Bacteria that require preformed folic acid are insensitive

Figure 7 shows the growth of *R.intestinalis* in the presence of the antibiotic Azithromycin. From this figure it can be seen that when compared to the control containing no antibiotics, *R.intestinalis* is not sensitive to Azithromycin. Since Azithromycin is a broad but shallow antibiotic (28) that treats only some gram positive and negative bacteria *R.intestinalis* may not fall into the scope of this antibiotic and therefore shows no sensitivity.

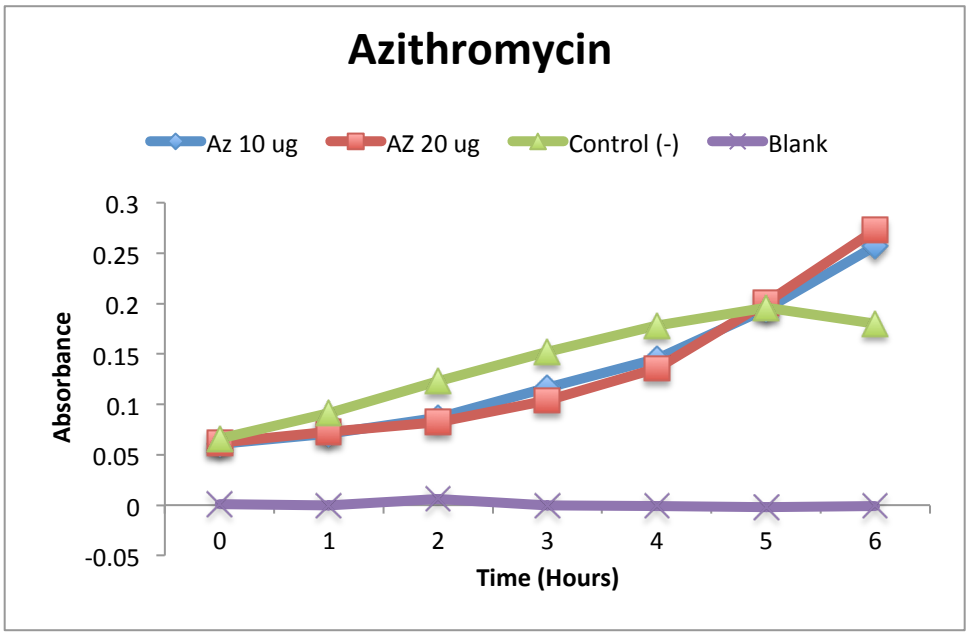

Figure 7 – Survivability of *R.intestinalis* in the presence of the antibiotic Azithromycin at 10 µg/mL and 20 µg/mL when compared to a negative control.

Figure 8 shows that *R.intestinalis* is not sensitive to Sulfamethoxzaole. Sulfamethoxzaole's mode of action is to prevent folic acid synthesis in bacteria that require synthesizing their own folic acid rather than utilizing their host's folic acid (29). It is likely that *R.intestinalis* does not synthesize folic acid and therefore falls outside of this antibiotics mode of action, although research has not yet been done to confirm *R.intestinalis'* mechanism for up taking folic acid.

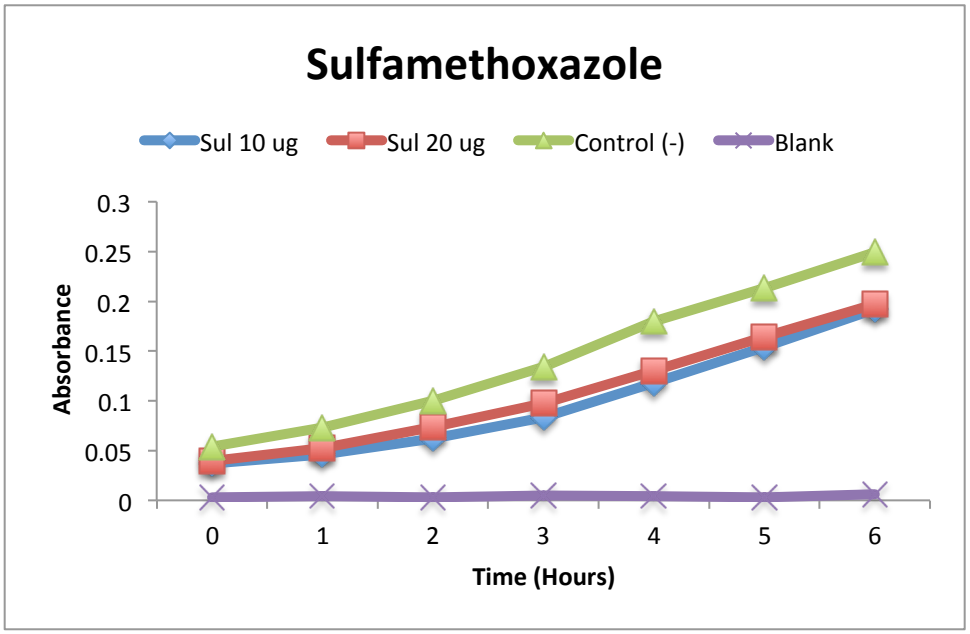

Figure 8 - Survivability of *R.intestinalis* in the presence of the antibiotic Sulfamethoxazole at 10 µg/mL and 20 µg/mL when compared to a negative control.

Figure 9-12 show the sensitivity of *R.intestinalis* in the presence of Oxytetracycline, Trimethoprim, Erythromycin, and Ciprofloacin, respectively. *R.intestinalis* was found to be sensitive to these antibiotics and increased growth was not observed. Oxytetracycline's mode of action is to affect the way bacteria synthesize and produce proteins (30). *R.intestinalis* was found to be the most sensitive to this antibiotic at both concentrations tested.

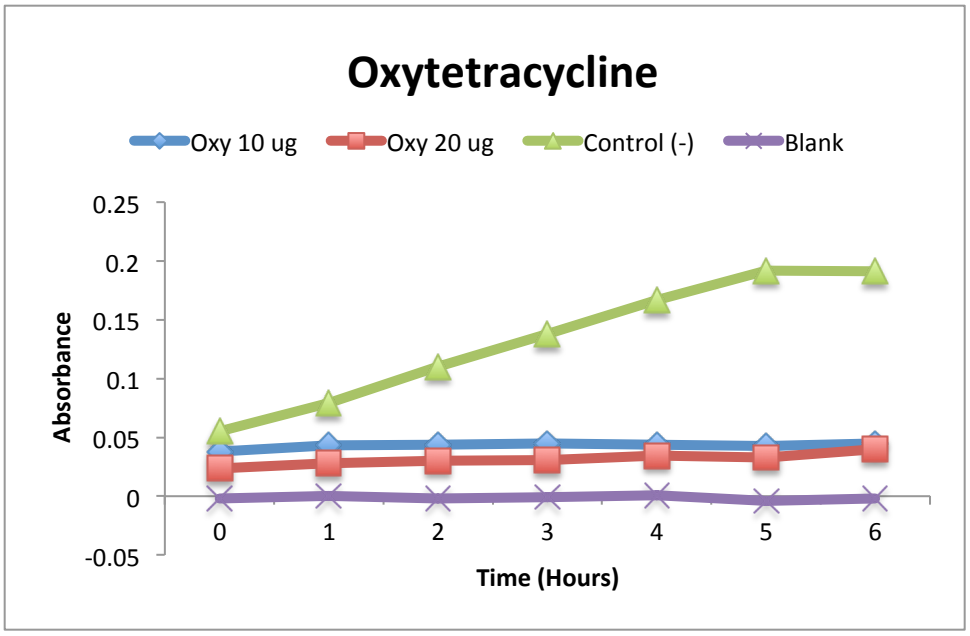

Figure 9 - Survivability of *R.intestinalis* in the presence of the antibiotic Oxytetracycline at 10 µg/mL and 20 µg/mL when compared to a negative control.

R.intestinalis was also found to be sensitive to the antibiotic Trimethoprim although it is not recommended for use on anaerobic bacteria. Upon further investigation, Trimethoprim interferes with the action of dihydrofolate reductase and the anaerobic bacteria that have been shown sensitive to Trimethoprim fall in different classes than *R.intestinalis (31).* As a result, anaerobic bacteria that fall into the class Clostridium may be sensitive to Trimethoprim's mode of action and as a result growth of *R.intestinalis* in its presence cannot be observed.

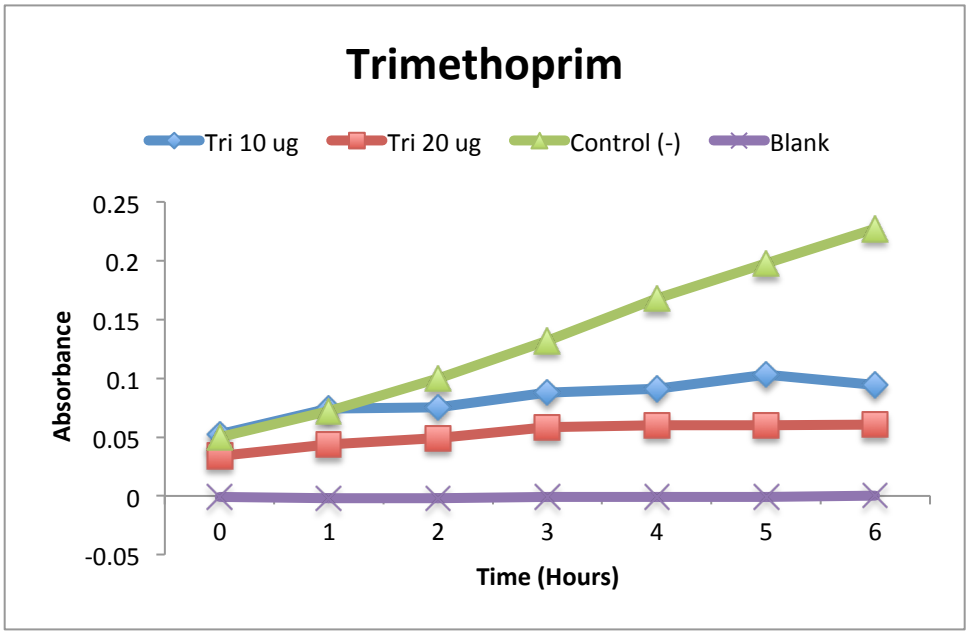

Figure 10 - Survivability of *R.intestinalis* in the presence of the antibiotic Trimethoprim at 10 µg/mL and 20 µg/mL when compared to a negative control.

Additionally, *R.intestinalis* was found to be sensitive to the antibiotic Erythromycin. Erythromycin mode of action is not yet understood, but it does show bacteriostatic activity (32). *R.intestinalis* may be sensitive to the bacteriostatic activity observed from Erythromycin and in turn is why increased growth is not observed when compared to the negative control although there is slight growth at 6 hours. Over time, without additional doses, surviving *R.intestinalis* may be able to overcome the bacteriostatic effects and begin to repopulate.

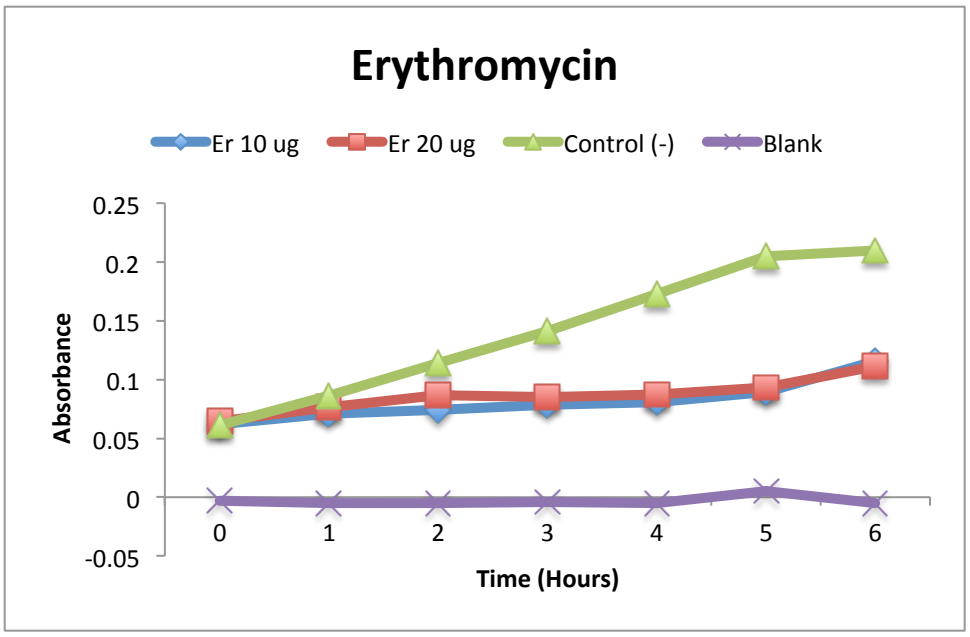

Figure 11 - Survivability of *R.intestinalis* in the presence of the antibiotic Erythromycin at 10 µg/mL and 20 µg/mL when compared to a negative control.

Finally, *R.intestinalis* was found to be sensitive to Ciprofloacin when compared to the negative control. Although *R.intestinalis* was found to be sensitive to Ciprofloacin, it did appear to have very slight growth in the beginning before no growth is observed. Ciprofloacin's mode of action is to inhibit the enzymes topoisomerase II and IV (33). Ciprofloacin's mode of action may take a couple generations of growth before taking full inhibitory effect on the *R.intestinalis* and may be why slight growth is seen in the beginning before no increased growth is obsereved.

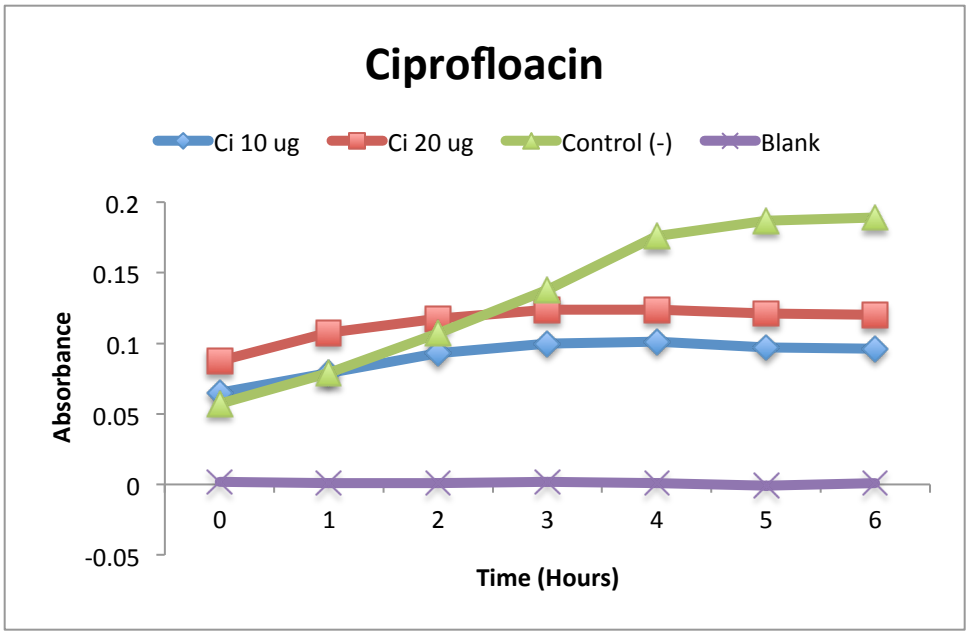

Figure 12 - Survivability of *R.intestinalis* in the presence of the antibiotic Ciprofloacin at 10 µg/mL and 20 µg/mL when compared to a negative control.

Generation Time and Growth Curve

Table 4 shows the generation time was found using the generation time equation from CFU count over four hours of growth. *R.intestinalis* generation time was found to be 30.5 minutes.

Table 3 – Generation time of *R.intestinalis* over 4 hours calculated using the generation time equation. Generation time found to be 30.5 minutes.

Time	CFU ($\log_{10}$)
0 hours	$10^{8.061}$
4 hours	$10^{10.443}$

log Change = 10.443 – 8.061 = 2.383

$$G = \frac{240 \text{ minutes}}{3.3\ (2.383)} = 30.5 \text{ minutes}$$

Figure 13 is the absorbance curve that was generated from absorbance readings plotted against their respective CFU counts. The equation generated can be used to find the CFU of *R.intestinalis* when and absorbance is found. The R^2 value is 0.99715 signifying that this model fits the data and is a good tool for finding CFU.

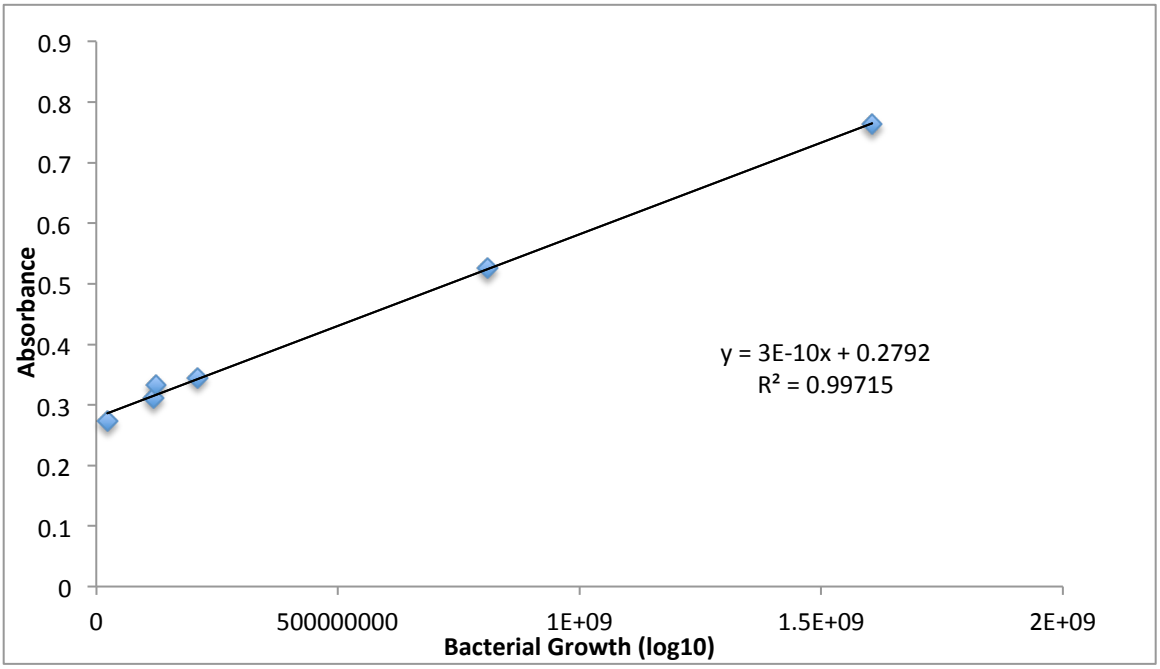

Figure 13 – Absorbance curve of *R.intestinalis.* R^2 value is 0.99715 showing model is a good fit of the data and can be used to find the CFY of *R.intestinalis* when absorbance is taken

Encapsulation

For *R.intestinalis* to be a successful probiotic it needs to survive the stomach and GI conditions in order to repopulate the gut. pH 2 did not show growth and because of this, encapsulation was done for protection. **Error! Reference source not found.** compared the growth of encapsulated freeze-dried and non-encapsulated freeze dried *R.intestinalis* after being exposed to physiological digestive conditions. Trial 1 shows no

significant difference in the growth of *R.intestinalis* when comparing encapsulated to not encapsulated. A second trial was conducted and similar results were seen, there was not increased survivability of the *R.intestinalis* in the encapsulated samples. From these results it can be hypothesized that *R.intestinalis* may grow in physiological digestive conditions without encapsulation. Since pH samples were not plated to check viability, it cannot be assumed that just because there was no growth in pH 2 that would make the *R.intestinalis* unviable. Further investigation into *R.intestinalis'* viability at low pH and survivability in physiological digestive conditions will need to be conducted to see the best vehicle for distributing *R.intestinalis* as a potential probiotic.

CONCLUSION

This study focused on the optimal growth, survivability, and storage conditions of a novel potential probiotics *R.intestinalis.* When SCFAs were added, *R.intestinalis* was found to grow the best at 66 mM acetate, but did show similar growth at 33 mM acetate with the addition of 9 mM propionate. The prebiotics guar gum and isomaltooligosaccharide showed similar growth to that of glucose. Survivability was examined and growth was observed in pH 6 – pH 9. *R.intestinalis* was found survive under low bile salt conditions but was not viable at concentration 5 g/L. Also, *R.intestinalis* showed no sensitivity to Azithromycin and Sulfamethoxazole. Encapsulation did not increase the survivability of the *R.intestinalis* when exposed to physiological digestive conditions and compared to samples that were not encapsulated. Further investigation into *R.intestinalis* and eventual animal experimentation may show the beneficial effects this novel bacterium could have as use in a probiotics.

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ABSTRACT

INVESTIGATION INTO *ROSEBURIA INTESINALIS* FOR OPTIMAL GROWTH AND SURVIVABILTY CONDITIONS FOR POTENTIAL PROBIOTIC USE

by

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May 2017

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Major: Nutrition and Food Science

Degree: Master of Science

Obesity and diabetes is an epidemic of increasing concern. Certain novel bacteria have been found to be an aid in reversing some of the mechanisms associated. This study focused on investigating the optimal stability conditions for *R.intestinalis* that can associate with an increase in growth. First, environmental conditions were tested to find the optimal conditions associated with each. Once growth conditions were found, generation time was calculated and various prebiotics were used to investigate a potential for additional growth. Next, common antibiotics were tested to see if the *R.intestinalis* could still show growth when exposed. Large batches of *R.intestinalis* were grown and freeze dried for storage and further experimental purposes. The freeze-dried *R.intestinalis* was encapsulated and reconstituted to check for viability. Finding the optimal conditions for growing *R.intestinalis* and using the encapsulation technique to protect the *R.intestinalis* from unfavorable environmental conditions could lead to the potential for *R.intestinalis* to become a probiotic that could help people suffering from adverse GI diseases and symptoms as well as decrease GSIS due to excess acetate.